December 14, 1972

Dr. F. Kisseljou
Biochemistry
D.I. Ivanovsky Virology Institute
Gamaleya Street 16
Moscow D98
U.S.S.R.

Dear Dr. Kisseljou:

Dr. Robert MacAllister has informed us of your interest in our techniques for detecting virus-specific nucleotide sequences in cell DNA. I am sending you a selection of reprints and preprints which will hopefully be of assistance. Our standard assay for Rous sarcoma virus-specific DNA in cells is currently done as follows:

Reagents

1) Cell DNA (at least 500 µg) prepared from at least $5 \times 10^7$ cells. Cells are suspended ($5 \times 10^6$-$10^7$ cells/ml) in 0.05 M Tris HCl, pH 8, 0.02 M EDTA, 0.1 M NaCl, lysed with 0.4% SDS and digested overnight at 37° C. with 500 µg/ml (predigested) pronase. The SDS concentration is adjusted to 1%, and two phenol extractions (gentle shaking at room temperature, no pipetting of DNA - it is poured) are done. Two volumes of ethanol are added to the aqueous phase, and the sample is stored at -15° C for at least one hour. The DNA is centrifuged at 2000 rpm for 15 minutes, gently resuspended in 0.01 M EDTA, 0.01 M Tris, pH 7.4 after thorough drying, and treated with 100 µg/ml of pancreatic ribonuclease for at least 3hhours (generally overnight) at 37° C. Phenol extractions (2) are repeated and the DNA is dialyzed for 2-4 days against several changes of 0.1X standard saline-citrate (SSC = 0.15 M NaCl, 0.015 M Na citrate). The optical density is determined at 260 and 280 (the 260/280 ratio should be greater than 1.8); if large quantities of DNA have been prepared, part is saved for network formation (to determine integration of viral genes - see Lepetit manuscript). The remainder is sheared to pieces about 200 nucleotides in length with a high pressure cell at 50,000 pounds per square inch. (Alternate available methods include depurination and sonication). The sheared DNA is then ethanol precipitated overnight (after a chloroform extraction to remove mineral oil from the pump line and addition of NaCl to 0.3 M) and (usually) passed over a G-50 sephadex column equilibrated with 0.6 M NaCl, 0.001 M EDTA, and 0.01 M Tris pH 7.4. The peak fractions are ethanol precipitated overnight and suspended in 0.003 M EDTA at a final concentration of greater than 5 mg/ml.

2) Labeled double stranded viral polymerase product is prepared as described in the manuscripts. If only the rapidly reassociating fraction is available due to low polymerase activity, high specific activity DNA (about $2.5 \times 10^7/\mu g$) usually labeled with all four deoxynucleotide precursors) is required for detection of a single copy of viral sequences with reasonable quantities (less than 0.5 mg) of cell DNA. If slowly reassociating DNA can be prepared, specific activities of $5 \times 10^6$ cpm/$\mu g$ are adequate due to the greater complexity (high $Cot_{1/2}$) of this material. (The higher complexity, of course, also indicates that more sequences are being measured with this DNA).

3) Hydroxyapatite (Biogel-DNA grade) should be standardized for separation of known single and double stranded DNA's in the presence of the amount of cell DNA present in each sample in a reassociation curve.

Procedure

1) Reaction mixtures usually contain 400 $\mu g$ of cell DNA and 2000 cpm of labeled polymerase product in .003 M EDTA in a final volume of 90 $\mu l$ in a well sealed glass conical tube (Kontes). The mixture is heated at 100° C for 2 minutes, 10 $\mu l$ of 4M PB (solution containing 2 M $Na_2HPO_4$ and 2 M $NaH_2PO_4$) is added, and the mixture is overlaid with mineral oil and incubated at 68° C. Six 15 $\mu l$ samples are taken at appropriate intervals (e.g., 1,3,8,24,48,72 hours) and placed in 0.01 M PB (2 ml). Samples are kept at 4° C until the conclusion of the experiment. Each experiment should include a heterologous cell DNA (e.g. salmon sperm or calf thymus) as a viscosity control.

2) Assays are performed with hydroxyapatite elution using 2 ml of a 10 Gm per 50 ml of 0.01 M PB mixture of hydroxyapatite. Elutions are performed at 60° C and consist of 3 ml and 2 ml elutions with 0.16 PB and 2 ml and 2ml with 0.40 M PB. OD260 is measured for the pooled .16 M and pooled .40 M sample to determine reassociation of the cell DNA, and the samples are then precipitated with a final concentration of 5% trichloracetic acid after addition of 75 $\mu g$ of calf thymus DNA as carrier.

3) Computations are performed by plotting reassociation of labeled polymerase product against the Cot value for the labeled DNA. Results are corrected according to single and double-stranded standards run over hydroxyapatite in each experiment. The conditions described here will cause at least a doubling of the reassociation rate (i.e., 2 fold lowering of the $Cot_{1/2}$) if one copy of the viral sequences are present per cell.

Thank you for your interest in our work. If I can be of further assistance, do not hesitate to write. I plan to be in Europe for a few weeks this summer and would welcome the opportunity to discuss our work further if appropriate financial and travel arrangements could be made.

Yours truly,

Harold E. Varmus, M.D.
Department of Microbiology

HEV:js
encl.